Supporting Information

Pattaradilokrat et al. 10.1073/pnas.0811430106

SI Text

SI Results. ***Physical location of* pyebl *in the* P. y. yoelii *genome.*** We were unable to locate *pyebl* in the *P. y. yoelii* genome, and our subsequent attempts to locate it in the *P. falciparum* genome database were also unsuccessful, because the *P. y. yoelii* contig, MALPY01471 (4.6 kb in length), in which *pyebl* (PY04764) is located (1), has no corresponding identifiable region in the *P. falciparum* genome. We next used the nucleotide sequence of *pyebl* in a BLASTN search of the genome of the related rodent malaria parasite *Plasmodium chabaudi chabaudi* clone AS. This resulted in the identification of the *P. c. chabaudi* contig containing an ortholog of *pyebl*, *P. c. chabaudi ebl*, PC300204.00.0 (*pcebl*). This gene was located on a large contig, contig003366 (775 kb in length) that allowed the identification of 2 closely linked genes upstream and downstream of *pcebl*, which are PC400152.00.0 and PC000605.0.2, respectively. BLASTP searching of the *P. falciparum* genome identified the orthologs of these flanking genes, PF14_0663 and PF14_0664, on *P. falciparum* chromosome 14. According to a genetic synteny map (2), their orthologous genes in *P. yoelii* are PY07221 and PY01695, which are predicted to be located at positions 1402 and 1407 kb, respectively, on *P. y. yoelii* chromosome 13, thus lying within the region found to be under strongest multiplication rate selection in the present study.

The physical linkage between *pyebl* and the 2 flanking genes, PY07221 and PY01695, was then tested by using long PCR. The results demonstrate that the genes PY07221 and PY01695, spanning approximately 8 kb of *P. yoelii* chromosome 13, lie approximately 3 and 4 kb away from the stop and start codons of *pyebl*, respectively (Fig. S3). From this data, *pyebl* was estimated to be located at position 1406 kb on *P. yoelii* chromosome 13, which lies approximately 120 and 200 kb from the 2 Pyrosequencing markers, 14-2968 and 13-0949, the 2 other 33XC markers that were most strongly reduced under growth selection (Fig. 3). The 33XC allele of *pyebl* itself was consistently reduced to less than 4% in the multiplication rate selected progeny (Table S2) and, in all of the selected progenies from both crosses, it was under stronger selection than any other locus observed in this study.

In addition, a ^{32}P-radiolabeled probe specific to *pyebl* hybridized on the PFGE chromosome blots for 17XYM and 33XC to a single band corresponding to the co-migrating *P. y. yoelii* chromosomes 13 and 14 (Fig. S2, lane 6), in agreement with the genetic mapping results described above.

SI Materials and Methods. ***Maintenance of rodent hosts and mosquitoes.*** Experimental mice were kept on water supplemented with 0.05% paraminobenzoic acid and fed on 41B rat and mouse maintenance diet (Harlan-Tekld) ad libitum. Animals were housed in polypropylene cages and kept at a constant 25 ± 1 °C on a 12-h light/dark cycle. Adult mosquitoes were supplied with a 10% glucose solution supplemented with 0.05% paraminobenzoic acid and were kept in a temperature and humidity controlled room (26 ± 1 °C, 80 ± 5% humidity) on a 12-h light/dark cycle. All experiments in this study were conducted in accordance with the Animals (Scientific Procedures) Act 1986 (UK).

Pyrosequencing (PSQ) assay conditions. An overview of the protocols to design and perform Pyrosequencing assays is published elsewhere (3). PSQ assays were designed by using the PSQ Assay Design Software Version 1.06 (Biotage AB). This requires the use of 2 oligonucleotides as PCR primers, one of which is biotinylated, and a sequencing primer close to the SNP. Pyrosequencing primers and the SNPs used in the present work are given in Table S3 (see below). The assays used here utilized the following conditions. PSQ PCRs were carried out in a 50-μL volume consisting of 1.5 mM $MgCl_2$, 0.3 μM each PSQ PCR primers, 1 mM dNTPs, 1 unit Immolase Polymerase (Bioline) in 1× Immobuffer (Bioline). PCR samples were thermocycled by using a Biometra T3000 by using an initial hot start (95 °C for 7 min), followed by 1 cycle of 95 °C for 3 min, 59 °C for 1 min, 72 °C for 1 min, followed by 40 cycles 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and followed by a final elongation step at 72 °C for 10 min. PCR products were run on 1.5% agarose gels. Each sample produced single amplicons of the predicted length. PCR products were, thereafter, subjected to a sequencing reaction with a corresponding PSQ sequencing primer by using a PSQ HS-956A instrument according to manufacturer's protocols.

Genetic crossing. Two genetic crosses were made between *P. y. yoelii* 17XYM and 33XC as follows. Each parasite clone was initially grown separately in donor mice. Parasites of the 2 clones were harvested from the donors and mixed to produce an inoculum in a proportion of 1:1. The mixture was inoculated i.p. at 1×10^6 parasitized RBCs per mouse into 3 CBA mice and 3 C57 mice to produce the first and second genetic crosses, respectively. These crosses will be referred to hereafter as cross 1 and cross 2. Four days after infection, when mean parasitemias were 20% and 6% in the CBA and C57 mice, respectively, and the presence of gametocytes of both sexes was confirmed microscopically, mice in each group were anesthetized and placed on 1 mosquito cage each (approximately 400 female *A. stephensi* mosquitoes per cage) before being humanely killed. Mosquitoes were then allowed to feed on the mice without interruption. Seven days after the blood meal, 6 female mosquitoes from each cage were dissected to examine for the presence of oocysts in mosquito midguts. For cross 1, 5 of 6 dissected mosquitoes were infected and had a mean of 33 oocysts per midgut. For cross 2, all 6 dissected mosquitoes were infected with a mean of 50 oocysts per midgut. Seventeen days after the initial blood meal, the surviving mosquitoes (200 and 359 mosquitoes for cross 1 and 2, respectively) were dissected, and the salivary glands (containing sporozoites) were removed. The glands were placed in 0.2–0.4 mL volumes of 1:1 fetal bovine serum/Ringer's solution (2.7 mM potassium chloride, 1.8 mM calcium chloride, 154 mM sodium chloride) and gently disrupted to release sporozoites. The suspensions were injected i.p. into groups of CBA mice in 0.1 mL aliquots to obtain blood stage parasites of the progeny of cross 1 and cross 2. The likely maximum numbers of recombinant lines in the uncloned progeny of each genetic cross were estimated (Table S5, see below), as previously described (4).

***Sequence analysis of* pyebl.** The full-length *pyebl* gene was amplified from genomic DNA prepared from blood stage parasites of *P. y. yoelii* as follows. PCR primers were 5′-CCTCCTGTTGCATAGTAGTATTGAT-3′ and 5′- TTTGATGAACCAAATGCATAGA-3′, corresponding to positions 1,407–1,431 and 4,232–4,209 of the genomic contig MALPY01471 of *P. y. yoelii* 17XNL deposited in the GenBank database under accession number AABL01001466 (1). PCRs were performed in a 50-μL volume consisting of 150-ng genomic DNA, 4 μM forward and reverse PCR primers, 2.0 mM $MgCl_2$, 1 mM dNTP, 5 units Bio-X-ACT DNA polymerase (short) (Bioline) and 1× Optibuffer (Bioline). PCR conditions were as follows: 1 cycle of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 68 °C for 4 min, and final extension at 72 °C for 10 min. All reactions were

performed on a Thermocycler (Perkin-Elmer). PCR products were analyzed by agarose gel electrophoresis and visualized by UV transillumination. Genomic DNA of each parasite clone produced single amplicons of the predicted length. The amplicons were purified with QIAquick PCR purification kit (Qiagen) and sequenced by using an ABI Prism BigDye Terminator ready-to-use reaction kit (version I; Applied Biosystem). The forward direction sequences were derived from the primers as follows: 5′-CCCCTTTATTTGTAGCATTTGG-3′, 5′-TTTTGTGATGCATCTGTTGGA-3′ and 5′-CCTTCACGTGCTGCATCTT-3′, corresponding to positions 2058–2079, 2603–2623, and 3365–3384 of the genomic contig MALPY01471 of *P. y. yoelii* 17XNL. The reverse direction sequences were derived from the primers as follows: 5′-ATTCAAATCCAACTCAAATTCC-3′, 5′-CGTGTAATACATATGAGGCATGG-3′ and 5′-TTCCAGACACAATAAAAATACCG-3′, corresponding to positions 2212–2192, 2912–2889, and 2458–2436 of the genomic contig MALPY01471 of *P. y. yoelii* 17XNL. DNA sequences of each parasite clone were visualized by using Edited View ABI automated DNA sequence viewer software (PerkinElmer ABI) and assembled manually to generate a full-length sequence of the *pyebl* gene, corresponding to positions 1561–4124 of the genomic contig MALPY01471 of *P. y. yoelii* 17XNL (1). The 2564-bp nucleotide sequences of the 4 parasite clones were aligned by using online sequence alignment software, provided by EMBL-EBI ClustalW (http://www.ebi.ac.uk/clustalw/).

Direct genomic mapping by PFGE and Southern hybridization. Host cell-free parasite pellets were prepared from the blood stages of *P. y. yoelii* 17XYM and 33XC, as previously described (5). The pellets were resuspended with an appropriate volume of phosphate buffer saline, pH 7.2 (Sigma) and mixed with an equal volume of 2.0% low melting temperature agarose (Sigma). The mixtures were added to $10 \times 5 \times 1.5$ mm^3 molds (Bio-Rad Laboratories) and allowed to set at 4 °C for 20 min. The solidified agarose blocks were then released into lysis buffer [0.5 M EDTA, 10 mM Tris-HCl, pH 9.5, 1% (vol/vol) N-lauroylsarcosine (Sigma), 0.2 mg/mL proteinase K (Sigma)] and incubated at 42 °C for 48 h with one change of the buffer after 24 h. Blocks were stored in 0.05 M EDTA, pH 8.0, at 4 °C until use.

PFGE was performed on the contour-clamped homogeneous electric field-DRII apparatus. Electrophoretic parameters were as follows: 100 volts, 360-s pulse time for 72 h; 100 volts, 720-s pulses for 24 h, and 0.7% agarose gel (6, 7). The gels were stained with ethidium bromide. Chromosomes were visualized by UV transillumination and photographed. The chromosomes were, thereafter, transferred onto Hybond *n*+ membrane (Amersham Biosciences), following the manufacturer's protocols.

Southern blots were incubated in QuikHyb hybridization solution (Stratagene) for 30 min before the addition of the ^{32}P-radiolabeled probe. Prehybridization and hybridization incubation temperatures were kept constant at 62 °C. The DNA probes specific to the 33XC AFLP markers, and *pyebl* were made by PRC and radiolabeled with approximately 3,000 Ci $mmol^{-1}$ α-[^{32}P]dATP (Amersham Biosciences) by using a Prime-It random primer labeling kit (Stratagene) and purified with a NucTrap probe purification column (Stratagene). PCRs were carried out in a 200-μL volume consisting of 300 ng genomic DNA of *P. y. yoelii* 33XC, 2.5 mM $MgCl_2$, 0.3 mM forward and reverse primers (as above), 400 μM dNTPs, 5 units *Taq* DNA polymerase in storage Buffer B (Promega) and 1× *Taq* polymerase 10× Buffer, magnesium-free (Promega). PCR conditions were as follows: 30 cycles of 94 °C for 45 s, 55–60 °C for 1 min, 72 °C for 1 min, and a final elongation step heating at 72 °C for 10 min. PCR products were run on 1.5% agarose gel. Each sample produced single amplicon of the predicted length, approximately 600 to 800 bp). Forward and reverse primers specific for AFLP marker 33XC AG01AG 17XYM were 5′-TGTTACAATATCCAAAAAGATGGTT-3′ and 5′-TCAAGAGATTAAAGATATTTGCAGATGA-3′, respectively. Forward and reverse primers specific for AFLP marker 33XC AG01TA 17XYM were 5′-GCAATTACACTTTTGTGTGAAA-3′ and 5′-TCCTTTACACATACTGCAACATT-3′, respectively. Forward and reverse primers specific for AFLP marker 33XC CA05TT 17XYM were 5′-CACAATTTCCCCATTTTTGTT-3′ and 5′-AAATGATGAAATTCAAAATGCTG-3′, respectively. Forward and reverse primers specific for AFLP marker 33XC CA05TA 17XYM were 5′-GACAAGGAAAGAAAAAGGAATCA-3′ and 5′-GGAAAGAGTGGCAAAAGTC-3′, respectively. Forward and reverse primers specific for AFLP marker 33XC AA01CT 17XYM were 5′-GTTTTCAATGGGTAGCCAAAAC-3′ and 5′-TTGCTGAAGATTGTAATCCCACT-3′, respectively. Forward and reverse primers specific for *pyebl* were 5′-CCCCTTTATTTGTAGCATTTGG-3′ and 5′-CGTGTAATACATATGAGGCATGG-3′, respectively. The probes were allow to hybridize to the preincubated blots for 1 h. Blots were washed twice for 15 min at room temperature with a 2× SSC buffer (0.3 M sodium chloride, 30 mM trisodium citrate dehydrate, pH 7.0), and washed twice for 30 min at 62 °C with prewarmed wash solution [0.2× SSC buffer and 0.1% (wt/vol) SDS] for a high-stringency wash. The washed blots were exposed to a Phosphor screen overnight before visualization with ImageQuant software. The blots were stripped and stored as recommended by the manufacturer's instructions.

***Long PCR for confirmation of physical location of* pyebl.** PCR assays were designed to determine possible physical linkage between *pyebl* and the 2 nearest flanking genes as predicted by genome database searching (see above). The genes for *P. y. yoelii* TATA element modulatory factor (PY07221) and acetyl-CoA carboxylase 1 precursor-related (PY01695) proteins, deposited in the GenBank under accession numbers XM_722945 and XM_724397 (1), respectively, were predicted to be the 2 nearest genes upstream and downstream of *pyebl*. During the course of this work, several sets of primers were designed to determine the location and orientation of *pyebl* relative to its 2 nearest flanking genes. The primers designed to specifically amplify a fragment containing the 5′ nucleotide sequence of *pyebl* and the 5′ reverse complement sequence of the gene PY07221 (the nearest gene upstream of the *pyebl*) were 5′-TCCATTTTCATTCTCTGTTTTCG-3′ and 5′-ATACAAGATCGCCCCATGTC-3′. The primers designed to specifically amplify a fragment containing the 3′ nucleotide sequence of the *pyebl* and the 5′ nucleotide sequence of the gene PY01695 (the nearest gene downstream of *pyebl*) were 5′-AACGATAGTGAAAATGCCAATAAA-3′ and 5′-TGGCCTCTCGTTTCTTTTTC-3′. The primers designed to specifically amplify a fragment containing the 5′ reverse complementary sequence of the gene PY07221 and the 5′ nucleotide sequence of the gene PY01695 were 5′-AAACAGTTTCATGGGAGTCTATAAAAG-3′ and 5′-CGTTTCTTTTTCTGCAAAATTATTGAT-3′. Other primers designed to amplify a gene fragment with gene orders or directions different from those which we described above gave no amplicons (data not shown).

PCRs were performed in a 50-μL volume consisting of 20 ng of genomic DNA of *P. y. yoelii* 17XYM and 4 units of BIO-X-ACT Long DNA Polymerase (Bioline) in 1× Optibuffer (Bioline) containing 1.5 mM $MgCl_2$, 1 mM dNTP, and 0.3 μL 100 μM Primer mix. The following cycling parameters were used: An initial hot start at 94 °C for 2 min and 58 °C for 1 min, conducted once, followed by 34 cycles of 94 °C for 30 s, 59 °C for 30 s, 68 °C for 20 min, and final elongation at 68 °C for 30 min. All reactions were performed on a Thermocycler (Perkin-Elmer). PCR products were analyzed by agarose gel electrophoresis and visualized by UV transillumination.

1. Carlton JM, et al. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 419:512–519.
2. Kooij TW, et al. (2005) A *Plasmodium* whole-genome synteny map: Indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* 1:e44.
3. Cheesman S, et al. (2007) Validation of Pyrosequencin for accurate and high throughput estimation of allele frequencies in malaria parasites. *Mol Biochem Parasitol* 152:213–219.
4. Pattaradilokrat S, Cheesman SJ, Carter R (2007) Linkage group selection: Towards identifying genes controlling strain specific protective immunity in malaria. *PLoS One* 2:e857.
5. Grech K, et al. (2002) Numerous, robust genetic markers for *Plasmodium chabaudi* by the method of amplified fragment length polymorphism. *Mol Biochem Parasitol* 123:95–104.
6. Owen CA, Sinha KA, Keen JK, Ogun SA, Holder AA (1999) Chromosomal organisation of a gene family encoding rhoptry proteins in *Plasmodium yoelii*. *Mol Biochem Parasitol* 99:183–192.
7. Khan SM, Jarra W, Bayele H, Preiser PR (2001) Distribution and characterisation of the 235 kDa rhoptry multigene family within the genomes of virulent and avirulent lines of *Plasmodium yoelii*. *Mol Biochem Parasitol* 114:197–208.

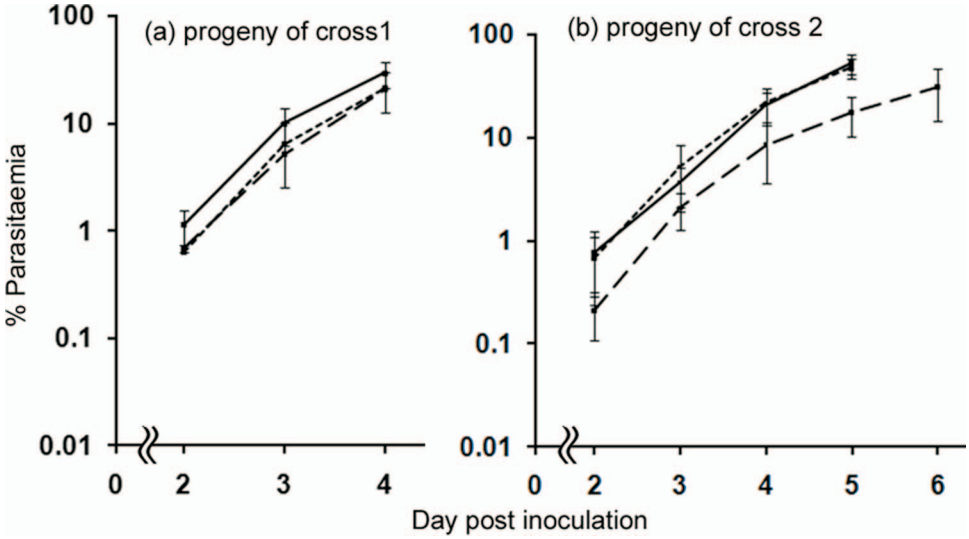

Fig. S1. Growth of the progeny of the 2 genetic crosses between *P. y. yoelii* 17XYM and 33XC in 3 successive blood-induced infections in mice. *a* and *b* show, respectively, total parasitemias of the progeny of the first and second crosses in their first blood passage (dashed line), second blood passage (dotted line), and third blood passage (solid line) after a sporozoite-induced infection from the uncloned progeny of each cross. The total parasitemias were measured by microscopic examination of thin blood smears stained with Giemsa's stain. Error bars give the 95% confidence intervals of the means.

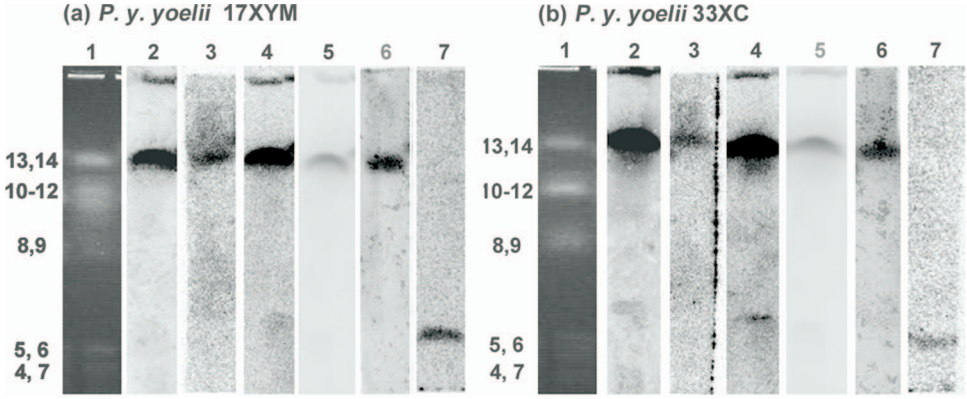

Fig. S2. Genomic locations of *P. y. yoelii* 33XC AFLP markers and the gene encoding the erythrocyte binding ligand (*pyebl*). Chromosome blots of 17XYM (*a*) and 33XC (*b*) were prepared by pulsed field gel electrophoresis and Southern blotting. The gels were stained with ethidium bromide. Lane 1 shows chromosomes stained with ethidium bromide and visualized by UV transillumination. The chromosome numbers (1, 2) are shown on the left-hand side of the gels in each panel. The chromosome blots were hybridized with ^{32}P-labeled DNA probes corresponding to the markers as follows: Lane 2, 33XC AG01AG 17XYM; lane 3, 33XC AG01TA 17XYM; lane 4, 33XC CA05TT 17XYM; lane 5, 33XC AA01CT 17XYM; lane 6, *pyebl*; lane 7, 33XC CA05TA 17XYM.

1. Owen CA, Sinha KA, Keen JK, Ogun SA, Holder AA (1999) Chromosomal organisation of a gene family encoding rhoptry proteins in Plasmodium yoelii. *Mol Biochem Parasitol* 99:183–192.
2. Khan SM, Jarra W, Bayele H, Preiser PR (2001) Distribution and characterisation of the 235 kDa rhoptry multigene family within the genomes of virulent and avirulent lines of Plasmodium yoelii. *Mol Biochem Parasitol* 114:197–208.

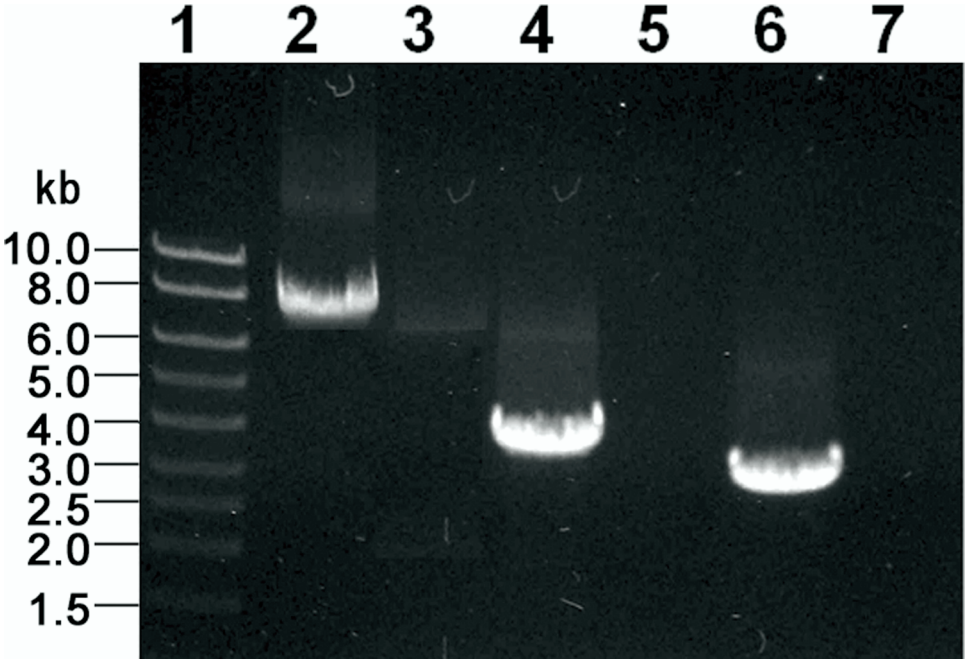

Fig. S3. Long PCR of a region on *P. y. yoelii* chromosome 13 containing the gene for erythrocyte binding ligand (*pyebl*). Lane 1, DNA size standard markers (Hyperladder I; Bioline). Lane 2, PCR products of the chromosomal portion between the 2 closely linked genes upstream and downstream of *pyebl*, PY07221 and PY01695, and, lane 3, of its negative control. Lane 4, PCR products of the region containing the genes PY01695 and *pyebl* and, lane 5, of its negative control. Lane 6, PCR products of the region containing the genes PY07221 and *pyebl* and, lane 7, of its negative control. Samples were analyzed on 0.7% ethidium bromide-stained agarose gel in 1× Tris-acetate EDTA buffer and visualized by UV transillumination.

(A)

```
A/C             ATGCAGTATTCGCTTATCACATGCAACCATGATAAGGTTACTAAAAAAAAAAAAAATATA 60
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             TATATATATAAATATATAAATGCATGATAAAATAAATGTGTGCAAAAATTAATATTATTC 120
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             AATACATTTATAAAATTGTTTGAATCATAGTAAAAGTATTAAATGCGATAAAATAAAAAA 180
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             ATAACTAATCAAATATTATTATTATATTTCATTTTTATATTATATTTTTTTCTTTATAGA 240
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             AAATCGTTAATTTATTAAAAAGAACATATGAATCTTTCCAATCTTTCCCTTTAAAAAATA 300
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             ATAGTGGAAATAGAATTTTATATGAACTTAAAGAATCTCTATATAACATTGAAAACGGAG 360
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------
                                                  401
A/C             AGACATGGGGCGATCTTGTATCAAAAGACAATGAAATTTTGGGGGGAAAAGATCATATAT 420
33XC            ------------------------------------------G-------------------
17X [NIMR]      ------------------------------------------T-------------------
17XYM           ------------------------------------------T-------------------
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(B)

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                                                                        2340
A/C             AAAATATAATAATGCTAGCCAACGAAAATGTATGCAATGGCAATCCAGGTTTAAAATACT 2340
33XC            ------------------------------------------------------------T
17X [NIMR]      ------------------------------------------------------------T
17XYM           ------------------------------------------------------------C

A/C             GTGAATCTATAAATAATAAATATTTTCATGGAACGTGTTCAAAAAGTCAAACCCAAAATC 2400
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             TTTGTTGCTCAATTTCAAATTATTGCATAAAATTTTTTAATATAAATTCAAAGAAATATT 2460
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             ATGATTGTATGAATGAAGAGTTTATGGCCCCAGATTATAACTGTTTTCAAAAAGAGAGCT 2520
33XC            ------------------------------------------------------------
17X [NIMR]      ------------------------------------------------------------
17XYM           ------------------------------------------------------------

A/C             ATTCAAGTAAGCGCCAATTTTTAGAAATTACATTGCTTCATTAA 2564
33XC            ---------------------------------------------
17X[NIMR]       ---------------------------------------------
17XYM           ---------------------------------------------
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Fig. S4. Multiple sequence alignment from the gene encoding erythrocyte binding ligand (pyebl) from the 4 cloned lines, A/C, 33XC, 17X [NIMR], and 17XYM, of *P. y. yoelii*. The (*A*) 5′ and (*B*) 3′ nucleotide sequences presented here are those that contain polymorphic residues (shown in red) between these lines; they cover positions 1–420 and 2281–2564 of the reference clone, 17XNL clone1.1, of *P. y. yoelii* (available in the TIGR database under the locus name PY04764 at http://www.tigr.org/tdb/e2k1/pya1/LocusNameSearch.shtml). All 3 slow-multiplying parasite lines were identical, but genetically distinct from the fast-growing parasite, 17XYM, at position 2340 in *pyebl*. The SNP at position 401 in *pyebl* does not segregate with the multiplication rate phenotype of these parasite lines. Our findings are, therefore, consistent with the findings of Otsuki and colleagues [Otsuki H, et al. (2009) Single amino acid substitution in *Plasmodium yoelii* erythrocyte ligand determines its localization and parasite virulence. *Proc Natl Acad Sci USA*, 10.1073/pnas.0811313106.] that the SNP at position 2340 in *pyebl* causes an amino acid substitution in region 6 of PyEBL that determine virulence and rbc selectivity in *P. y. yoelii*. (−) indicates identical nucleotide between the 4 lines analyzed. The full-length sequences of the *pyebl* gene from 17XYM, 17X [NIMR], 33XC, and A/C of *P. y. yoelii* were deposited in the GenBank database under the accession numbers FJ610238, FJ610239, FJ610240, and FJ610241, respectively.

Table S1. Relative intensity indices (RIIs) and predicted physical locations of clone 33X AFLP markers strongly reduced following multiplication rate selection

Name of *P. y. yoelii* 33XC AFLP marker under strong selection	RIIs of markers in the first uncloned progeny			RIIs of markers in the second uncloned progeny			*P. y. yoelii* contig* with the highest sequence homology to marker	Physical locations of *P. y. yoelii* orthologues in the *P. falciparum* genome†	Predicted physical location in the *P. y. yoelii* genome‡
	first passage	second passage	third passage	first passage	second passage	third passage			
33XC AG01AG 17XYM	0.073	0.067	0.065	0.096	0.077	0.038	MALPY02017	PF14-1946	PY13-0502
33XC AG01TA 17XYM	0.104	0.056	0.045	0.103	0.067	0.089	MALPY01629	PF14-2476	PY13-1035
33XC CA05TT 17XYM	0.01	0.01	0.01	0.045	0.061	0.033	MALPY00279	PF13-0949	PY13-1602
33XC CA05TA 17XYM	0.255	0.01	0.03	0.198	0.207	0.144	MALPY02313	No orthologue	Unknown
33XC GT02TT 17XYM	0.07	0.038	0.027	0.314	0.316	0.199	MALPY00154	No orthologue	Unknown

*Contigs of *P. y. yoelii* [Carlton JM, et al. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 419:512–519] could be accessed through the NCBI Nucleotide database website (http://www.ncbi.nlm.nih.gov/sites/entrez?db = nuccore).

†Numbers after 'PF' indicate the *Plasmodium falciparum* chromosome number followed by distance along the chromosome in kilobase pairs.

‡Numbers after 'PY' indicate the *Plasmodium yoelii* chromosome number followed by predicted distance on the chromosome in kilobase pairs, according to conserved genetic synteny between *P. falciparum* and *P. y. yoelii* [Kooij TW, et al. (2005) A *Plasmodium* whole-genome synteny map: Indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* 1:e44].

Table S2. Proportions of the progeny of the 2 genetic crosses between 17XYM and 33XC carrying the 33XC allele at Pyrosequencing marker locations on chromosome 13

Pyrosequencing markers	Physical locations of the orthologues of markers in the P. falciparum genome*	Predicted physical location in the *P. y. yoelii* genome†	Proportion of the 33XC allele in the first uncloned progeny			Proportion of the 33XC allele in the second uncloned progeny		
			first passage	second passage	third passage	first passage	second passage	third passage
14-3066	PF14-3066	PY13-0052	0.106	0.087	0.066	0.208	0.19	0.178
14-1793	PF14-1793	PY13-0349	0.062	0.048	0.042	0.163	0.163	0.154
14-1946	PF14-1946	PY13-0502	0.117	0.017	0.047	0.168	0.189	0.144
14-2211	PF14-2211	PY13-0767	0.057	0.014	0.049	0.139	0.134	0.124
14-2476	PF14-2476	PY13-1035	0.023	0.01	0.01	0.102	0.092	0.096
14-2708	PF14-2708	PY13-1264	0.136	0.14	0.101	0.086	0.101	0.066
14-2805 (*pyebl*)‡	PF14-2805	PY13-1406	0.039	0.01	0.01	0.022	0.01	0.01
14-2968	PF14-2968	PY13-1524	0.01	0.023	0.023	0.024	0.04	0.015
13-0949	PF13-0949	PY13-1602	0.01	0.01	0.01	0.043	0.039	0.032
13-1191	PF13-1191	PY13-1850	0.442	0.425	0.322	0.172	0.192	0.212
13-1950	PF13-1950	PY13-2609	0.285	0.328	0.307	0.547	0.557	0.561

*Numbers after PF indicate the *Plasmodium falciparum* chromosome number followed by distance along the chromosome in kilobase pairs.

†Numbers after PY indicate the *Plasmodium yoelii* chromosome number followed by predicted distance on the chromosome in kilobase pairs, according to conserved genetic synteny between *P. falciparum* and *P. y. yoelii* [Kooij TW, et al. (2005) A *Plasmodium* whole-genome synteny map: Indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* 1:e44] .

‡The 33XC allele of the gene for erythrocyte binding ligand protein (*pyebl*) is measured using marker 14-2805.

Table S3. Summary of Pyrosequencing (PSQ) primers and single nucleotide polymorphisms (SNPs) analyzed in the present work

Pyrosequencing markers	Pyrosequencing PCR primers, 5′ biotin to 3′	Pyrosequencing PCR primers, 5′ to 3′	Pyrosequencing sequencing primers, 5′ to 3′	SNP category†
AMA-1*	GGAAATGCCAATCCTCCACTT	AGGTGCATGGTTCTGGTATAAGAG	GGTATAACTATTCAAAATTC	C/A
14-3066	AACCTCCCTGAAAAACATTTAA	AACTGCAGAAAATTAAAGAGAACA	AAAAAATAAAAATCACAAAA	G/C
14-1793	AATGAACCCAAGTGTTACAGAATG	CTCCAATAAGCATTTTTGATGTGT	AAAATGTAAAATTATTTCGA	A/C
14-1956	TTCACTATTTTCCACACCCTTTAA	GAACCATTGAAAAAAATGCATAA	TTTGGAAATAATTTTATTGG	G/A
14-2211	GAAAAGCATGTCAAGCCTCTATCT	AGTTCGAAAATCAAAACAGTGAAA	ATATTCCACATTTTCGC	C/T
14-2478	TGTTGCAATGTTTGGTTTAAAAG	TTTTGCATCAAGGGAATTGTG	TCAAGGGAATTGTGA	A/G
14-2708	GCATTGTTAAATGAGGAAGATGC	TCAGCCAATTCAGGCCAATA	AATTCAGGCCAATATATATC	G/T
14-2805	TTGGGTTTGACTTTTTGAACACGT	AATGTATGCAATGGCAATCCAG	GCAATCCAGGTTTAAAATAC	C/T
14-2968	TTTCCATGTGATCAAGAAGATCG	TAAGGAGGGGGGGTTTCTTGTAAT	TTTCCTGGGTCTATAAGAG	C/A
13-0949	TTATTTGGTTGTTTCCTGAATATG	AATGATGCAATATATAGTGGGAAT	TGGGAATAATAACGCAG	C/T
13-1191	CCCTCCTAATACTATTGCGCATAC	GGCAGGAGATTTAGAGCACACT	GCTATGTGCGTGTCG	G/A
13-1955	AAATTATCTCGGATCGCAGAAAT	GGTAAAATTCAACTCGAATCATCA	CATCATTCTGGTTTTGATAC	T/A

*A PSQ assay designated AMA-1, located on *P. y. yoelii* chromosome 9, was used for measuring proportions of parasites carrying the 17XYM and 33XC alleles of the gene for Apical Membrane Antigen-1 (AMA-1) in the mixed cloned infection in mice. The other PSQ assays were designed to quantify proportions of parasites carrying the 17XYM and 33XC alleles of specified genes on *P. y. yoelii* chromosome 13 in the uncloned progeny of the genetic crosses between 17XYM and 33XC (see *Materials and Methods*).

†SNPs identified in cloned lines 17XYM and 33XC, respectively.

Table S4. The origin and characteristics of lines of the rodent malaria parasite *Plasmodiium yoelii yoelii* used in the present study

Cloned lines of *P. y. yoelii*	Origin	Year of cloning	Blood stage growth rate	GPI	Response to PYrimethamine
17XYM	isolate 17X	1974	fast	GPI-1	sensitive
17X(NIMR)	isolate 17X	1967–1978?	slow	GPI-2	sensitive
33XC	isolate 33X	1973	slow	GPI-2	sensitive
A/C	Progeny of a cross between 17XA and 33XC	1973	slow	GPI-2	resistant
604	Progeny of a cross between 17XYM and A/C	1975	fast	GPI-2	resistant
605	Progeny of a cross between 17XYM and A/C	1975	fast	GPI-2	sensitive
606	Progeny of a cross between 17XYM and A/C	1975	slow	GPI-1	sensitive
611	Progeny of a cross between 17XYM and A/C	1975	slow	GPI-1	sensitive
645	Progeny of a cross between 17XYM and A/C	1975	fast	GPI-1	resistant
648	Progeny of a cross between 17XYM and A/C	1975	slow	GPI-2	sensitive

Line 33XC is a slow-multiplying clone of *P. y. yoelii* isolate 33X [Walliker D, Carter R, Morgan S (1973) Genetic recombination in *Plasmodium berghei*. *Parasitology* 66:309–320]. Line 17X(NIMR) is a slow-multiplying clone of *P. y. yoelii* isolate 17X [Pattaradilokrat S, Cheesman SJ, Carter R (2008) Congenicity and genetic polymorphism in cloned lines derived from a single isolate of a rodent malaria parasite. *Mol Biochem Parasitol* 157:244–247]. Line 17XYM is a fast-multiplying clone of *P. y. yoelii* isolate 17X [Walliker D, Sanderson A, Yoeli M, Hargreaves BJ (1976) A genetic investigation of virulence in a rodent malaria parasite. *Parasitology* 72:183–194] that appears to have undergone a dramatic enhancement in multiplication rate and pathogenicity following the removal of a stabilate from liquid nitrogen storage [Yoeli M, Hargreaves B, Carter R, Walliker D (1975) Sudden increase in virulence in a strain of *Plasmodium berghei yoelii*. *Ann Trop Med Parasitol* 69:173–178]. Line A/C is a slowly multiplying clone of the progeny of a genetic cross between *P. y. yoelii* lines 17XA and 33XC [Walliker D, Sanderson A, Yoeli M, Hargreaves BJ (1976) A genetic investigation of virulence in a rodent malaria parasite. *Parasitology* 72:183–194]. Fast-multiplying clones, 604, 605, and 645, and slowly multiplying clones, 606, 611, and 648, were independently cloned from the progeny of the genetic cross between *P. y. yoelii* 17XYM and A/C [Walliker D, Sanderson A, Yoeli M, Hargreaves BJ (1976) A genetic investigation of virulence in a rodent malaria parasite. *Parasitology* 72:183–194]. GPI, glucose phosphate isomerase.

Table S5. The parameters used to estimate the likely maximum number of independent recombinant lines present in the pooled progeny of the 2 genetic crosses between clones 17XYM and 33XC of *Plasmodium yoelii yoelii*

Parameters	Genetic cross 1	Genetic cross 2
No. of mosquitoes dissected for oocysts (no. of infected mosquitoes)	6(5)	6(6)
Average no. of oocysts per midgut	33	50
No. of mosquitoes dissected for sporozoites	200	359
Predicted no. of oocysts represented	6600	17950
Predicted no. of recombinant lines	13200	35900

Table S6. A summary of primers used in identifying single nucleotide polymorphisms and/or insertion or deletion (indel) mutations between clones 17XYM and A/C of *Plasmodium yoelii yoelii*, PCR reactions, and cycling parameters

Physical location of the markers in *P. y. yoelii**	Orthologous locus in the *P. falciparum* genome†	Sequencing primer (forward direction) (5′ to 3′)	Sequencing primer (reverse direction) (5′ to 3′)	Sequence length, bp	Total no. of SNP	Total no. of indel mutation site	Single nucleotide polymorphism‡ and insertion or deletion mutation
PY13-0052	PF14-3066	GGTACACGTGAAGATCGTTT	TGTTTCGTTGCTTCATTTGC	701	2	0	G/C, C/T
PY13-0350	PF14-1793	CGCTGAAAATGGTGACAATG	CGGTAACAAAATTAACAAAATCCA	720	3	0	T/C, G/C, T/C
PY13-0502	PF14-1953	TTGTTACAATATCCAAAAAG-ATGGTT	TCAAGAGATTAAAGATATTGCAGATGA	357	0	1	[GAAGTTGGGAAGTTGGG] Insertion confined to A/C
PY13-1032	PF14-2478	GCAATTACACTTTTGTGTGAAA	TCCTTTAGAGATACTGCAACATT	326	0	1	[GTTATTTTT] Insertion confined to A/C
PY13-1406 (*pyebl*)§	PF14-2805	CCTCCTGTTGCATAGTAGTATTGAT	TTTGATGAACCAAATGCATAGA	2561	2	0	T/G, C/T¶
PY13-1602	PF13-0949	CACAATTTTCCCCATTTTTGTT	AAATGATGAAATTCAAAATGCTG	442	2	1	T/C, T/C, [TAACACTTCTTTGGCATTTTCTAA-CACTTCTTTGGCATTTTC] Insertion confined to 17XYM
PY13-1705	PF13-1046	AAAGGGGAAAAAGGGAATCA	ACATTCCGGTGTTCCAAAAA	670	4	0	G/A, A/G, A/G, C/A
PY13-1850	PF13-1191	TGCGATAAAAGAAGAAAATGGA	TTGGTGTAGTTCCCTTTGGA	707	3	0	A/T, G/A, C/T
PY 13-2609	PF13-1950	TGCATAAAATGCCAAAATGG	CAGGCTGCTACTTCGATTCC	583	1	0	T/A

PCR reactions were carried out in a 50-μl volume consisting of 1.0 mM $MgCl_2$, 0.2 μM each PCR primers, 0.5 mM dNTPs, 1 unit *Tag* DNA polymerase (Promega) in 1× Mg-free *Taq* DNA Polymerase buffer (Promega). PCR samples were thermocycled by using a Biometra T3000 with an initial hot start (95 °C for 7 min), followed by 1 cycle of 95 °C for 3 min, 60 °C for 1 min, 72 °C for 1 min, followed by 30 cycles 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and followed by a final elongation step at 72 °C for 10 min. PCR products were run on 1.0% agarose gels. Each sample produced single amplicons of the predicted length. PCR products were, thereafter, purified by using a QIAGEN PCR purification kit and sequenced by using a Big Dye Terminator (Applied Biosystems).

*Numbers after letters PY indicate *P. y. yoelii* chromosome, followed the position on chromosome in kb. The *P. y. yoelii* chromosomal designation for each locus was extrapolated from the genetic synteny map between *Plasmodium falciparum* and *P. y. yoelii* [Kooij TW, et al. (2005) A *Plasmodium* whole-genome synteny map: Indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* 1:e44].

†Numbers after letters 'PF' indicate *P. falciparum* chromosome, followed the position on chromosome in kilobase pairs.

‡SNPs identified in cloned lines 17XYM and A/C, respectively.

§Marker PY13-1406 (or *pyebl*) is an exception (see *SI Text* for the detailed method).

¶See also Fig. S4.